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Macrophage Migration Inhibitory Factor: Isolation from Bovine Brain

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Abstract—The purification of macrophage migration inhibitory factor (MIF) from bovine brain cytosol and its partial characterization are reported. A rapid and relatively simple method for MIF isolation was developed based mainly on size-exclusion chromatography on Toyopearl TSK polymer having a tendency to adsorb MIF as compared to elution of other proteins with similar molecular weights. The method gives a high yield of MIF (0.1 mg homogenous protein per g wet tissue). The retardation is conveniently utilized to achieve good separations of MIF from other proteins of similar molecular weights. The isolated protein was identified as MIF by SDS-electrophoresis, immunoblotting, sequencing of the N-terminal amino acid residues, and also by determination of keto–enol tautomerase activity that is characteristic of MIF with *p*-hydroxyphenylpyruvic acid as a substrate.

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Key words: macrophage migration inhibitory factor, gel filtration, keto–enol tautomerase

Macrophage migration inhibitory factor (MIF) is a cytokine that was discovered about 40 years ago. MIF was identified as a factor produced by activated T-lymphocytes due to its ability to inhibit *in vitro* macrophage migration [1, 2]. But biochemical studies on MIF were started only about 25 years later, after a recombinant human MIF protein and monoclonal anti-MIF antibody had been prepared [3]. Afterwards MIF was "rediscovered" as a hormone of the adenohypophysis [4] and a mediator of the organism's system response to stress [5, 6]. The involvement of MIF at all levels of the stress-induced functioning of the hypothalamus–hypophysis–adrenal system, regulation of MIF expression in the hypophysis under the influence of hypothalamic corticoliberin, and its release from the hypophysis together with adrenocorticotropic hormone (ACTH) [5, 7, 8] confirm the neuroendocrine role of MIF.

A low-molecular-weight highly conserved protein, MIF (12.5 kD) is widely expressed in various biological objects from bacteria to mammals. Its greatest amount is found in macrophages and different parts of the brain. *In vivo* and *in vitro* studies on the immunological role of MIF have shown it to be a cytokine which increases inflammatory reactions and inhibits antiinflammatory and immunosuppressive effects of glucocorticoids, mainly of dexamethasone and hydrocortisone, in picomolar concentrations [9, 10]. Glucocorticoids promote MIF release from cells, and this process is accompanied by an increase in the expression of such cytokines as interleukins (IL-1β, IL-2, IL-6, IL-8), tumor necrosis factor-α, and interferon-γ [10, 11]. A further increase in the concentration of glucocorticoids usually observed on exposure to stress is not associated with the counteracting effect of MIF, and its secretion is prevented, suggesting the fine regulation of the organism under stress conditions.

The role of MIF is now actively investigated in pathogenesis of neurodegenerative diseases [12], viral infections [13], various inflammations [10], and tumors [14, 15]. Involvement of MIF has been shown in cell proliferation [16], differentiation [17], and apoptosis [18]. MIF is also shown to be an enzyme possessing keto–enol tautomerase and protein oxidoreductase activities [19-22].

But although MIF is rather well studied, many aspects of its activity seem contradictory. In particular, the most interesting for biology and medicine regulation of MIF secretion under the influence of glucocorticoids observed on rodent cells was not found in studies on

Abbreviations: MIF) macrophage migration inhibitory factor; HPLC) high performance liquid chromatography; TFA) trifluoroacetic acid; β-ME) β-mercaptoethanol.

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human MIF. Moreover, neither exogenous glucocorticoids nor stimulants of ACTH secretion changed the MIF concentration in human blood [23]. No interrelation between the enzymatic and biological activities of MIF was revealed by a comprehensive structural–functional analysis of MIF [24-26]. Biochemical mechanisms of MIF involvement in pathogenesis of various diseases are not clear.

Genetic analysis has shown the existence of a single gene of human MIF and of several genes of mouse MIF [27, 28]. MIF has been demonstrated to have genetic polymorphism, which correlates with the human predisposition to some autoimmune and inflammatory diseases [8, 10]. The comparison of MIF polymorphic variants makes it possible to confront specific combinations of genetic changes in healthy and diseased persons and reveal the involvement of MIF in pathogenesis of the disease. Thus, functional polymorphism, which determines the high or low expression of MIF, is supposed to influence the aggravation or inhibition of development of rheumatoid arthritis and other inflammatory processes.

The amino acid sequences of the human and mouse MIFs have a 89% homology, but MIF is not homologous to other known proteins. Not only the primary structure of MIF is unique, but also its tertiary structure, which is not inherent in any known cytokine or pituitary hormone [29]. At physiological concentrations MIF forms an oligomeric structure, which is a mixture of monomers, dimers, and trimers [30], with dimer and monomer predominance in human and bovine MIF, respectively. However, different approaches give different data about the redistribution of protomers in the oligomeric structure of MIF.

We recently found a new property of MIF [31, 32]. Based on its structural–functional characteristics, MIF was supposed to be involved in biochemical mechanisms of the organism's protective reactions under heat shock, which causes aggregation and denaturation of labile proteins. In the *in vitro* test system based on kinetics of thermal aggregation of model protein substrates (glycogen phosphorylase *b* from rabbit skeletal muscle and malate dehydrogenase from porcine heart), MIF displayed chaperon-like activity manifested in the inhibition of the aggregation of the substrate.

It seems that the multifunctional nature of MIF in different systems is determined by existence of its different isoform and oligomeric structures as a result of post-translational modifications or conformational changes. Therefore, further studies on the features and action mechanisms of MIF seem reasonable, as well as elaboration of approaches for its isolation from different sources.

This work presents the results of studies on MIF isolated from bovine brain by a new original method, which provides high yield of homogenous protein. Different methods of MIF isolation are compared.

MATERIALS AND METHODS

Materials. Acetonitrile (HPLC grade) was from Merck (Germany); trifluoroacetic acid (TFA) (sequence grade) from Knauer (Germany); molecular weight standards, Tris, DEAE-Servacel, Immobilon polyvinyl difluoride membranes (PVDF), conjugate of rabbit antibodies to goat IgG with horseradish peroxidase, and *p*-hydroxyphenylpyruvate (*p*-HPP) from Sigma (USA); light sensitive Clear blue X-ray film from Pierce (USA); ECL-kit from Amersham Life Science (USA); acrylamide, N,N′-methylenebisacrylamide, and ammonium persulfate from Serva (Germany); Toyopearl HW-55 from Toyo Soda (Japan); polyclonal anti-human MIF antibody (Catalog No. AB-289-PB) from R&D Systems (England); other reagents were of chemical or analytic grade; water was deionized with a Milli Q System apparatus (Millipore, USA).

Preparation of bovine brain extract. Bovine brain obtained from a slaughterhouse was frozen and stored for a long time at −70°C. Before the isolation, the tissue was thawed and homogenized in a Waring blender type homogenizer in 100 mM Tris-HCl buffer (pH 7.8) (3 : 1) containing 100 mM KCl, 5 mM β-mercaptoethanol (β-ME), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10,000*g* for 30 min, and the supernatant was centrifuged at 60,000*g* for 1 h in an L7 centrifuge (Beckman, USA). The secondary supernatant was heated in a water bath at 58°C for 20 min and then centrifuged at 60,000*g* for 1 h. The supernatant was salted out with ammonium sulfate to the final saturation of 80%. The resulting suspension was stored for a long time at 4°C.

Size-exclusion chromatography. After centrifugation of an aliquot of the protein suspension in ammonium sulfate at 12,000*g* for 20 min, the precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.0) and applied onto a column with Toyopearl HW-55 (1.6 × 70 cm) equilibrated with the same buffer. The column was eluted at the rate of 0.6 ml/min. The MIF-containing fractions were determined by the tautomerase activity, and then they were subjected to SDS-electrophoresis, immunoblotting, and microsequencing.

N-Terminal microsequencing. Automated microsequencing by the Edman method was performed using a 473 A sequencer (Applied Biosystems, Germany) equipped with an online system for analysis of phenylthiohydantoin derivatives. After electrophoresis and immunoblotting on PVDF membranes, the MIF-containing bands were cut out and the protein was microsequenced by the standard procedure directly from the membrane.

Electrophoresis and immunoblotting. Electrophoresis of protein fractions purified by chromatography was performed by the Laemmli method [33] in 12% polyacrylamide gel in the presence of SDS at 360 V, 35 mA. The

protein was developed with Coomassie Brilliant Blue G-250 or silver staining.

After electrophoresis, MIF was identified by immunoblotting. The proteins were transferred onto nitrocellulose or PVDF membranes, and the spots were developed using an ECL kit (Amersham Life Science).

The keto–enol tautomerase activity of MIF. The enzymatic activity of MIF was determined by the rate of enol isomer production from keto isomer of *p*-hydroxyphenylpyruvic acid (*p*-HPP) used as a substrate [34, 35]. *p*-HPP was dissolved in 50 mM ammonium acetate (pH 6.0) and preincubated at 4°C for 12 h. In a quartz cuvette containing 0.435 M boric acid (pH 6.2), varied concentrations of *p*-HPP were added and MIF fractions (10-100 nM) were eluted from the column. Increase in absorption at 330 nm was determined at room temperature with a DU 650 spectrophotometer (Beckman).

Reversed-phase high performance liquid chromatography (HPLC). To identify the proteins eluted from the column, aliquots of the protein peak were filtered through a 0.45-μ filter (Sartorius, Germany), and the filtrate was subjected to reversed-phase HPLC on a C8 Aquapore RP 300 column (4.6 × 220 mm) (Brownlee Labs, USA) or on a C18 column (4.6 × 220 mm) (Vydac, USA) equipped with a precolumn (3.2 × 15 mm) using an HPLC system chromatograph (Gilson, France). The column was eluted with a linear gradient (0.1-0.08% TFA in acetonitrile (0-70%)) at the rate of 0.5 ml/min. The protein peaks detected by absorption at 214 nm were collected and dried in a vacuum Speedvac Drier concentrator (Savant, USA).

The protein concentration was determined as described in [36].

RESULTS AND DISCUSSION

For isolation of homogenous MIF a relatively easy approach was developed, which includes homogenization of the brain tissue, centrifugation, incubation of the supernatant at 58°C for 20 min, and the subsequent salting out of the supernatant with ammonium sulfate to the final saturation of 80%. This method allowed us to prepare a large amount of the salted out protein, which could be stored for at least six months at 4°C without loss of activity. Thus, for routine experiments the brain tissue could be stored at –70°C for as long as a year, 150 g of wet tissue was used for homogenization, and the salting out resulted in ~400 ml of suspension. When the homogenous protein was required, aliquots of the suspension (10 ml) were used, i.e., centrifuged, the precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.0), and the sample fractionated on a column with Toyopearl HW-55. The MIF-containing fractions were determined by the tautomerase activity with *p*-HPP as the substrate.

MIF was eluted from the column in fractions from 105 to 115 ml (Fig. 1, curve *3*). Using the molecular weight standards, MIF was shown to be retained on the Toyopearl HW-55 column (1.6 × 70 cm) and be eluted later than proteins with the corresponding standard molecular weight (Fig. 2). MIF binding with the sorbent and its delayed elution were successfully used for optimization of chromatography and isolation of MIF in a homogenous state. Because MIF is a thiol-dependent enzyme [21, 29, 34], the peak of its tautomerase activity

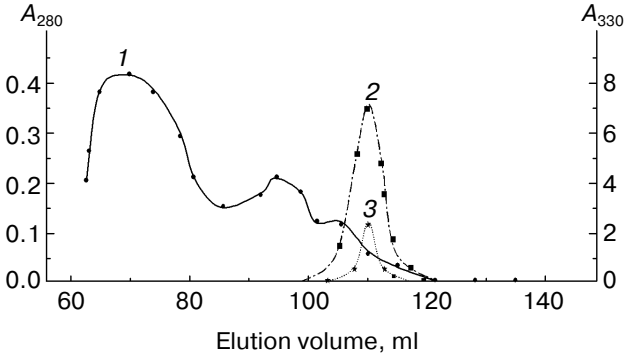

Fig. 1. Gel filtration of a partially purified extract of bovine brain cytosol on a Toyopearl HW-55 column (1.6 × 70 cm). *1*) Protein elution profile; *2*) keto–enol tautomerase activity using 20 mM Tris-HCl buffer (pH 7.0) containing 2 mM β-ME as the eluent; *3*) the same in the absence of β-ME. On the left ordinate the absorption at 280 nm and on the right ordinate the absorbance (×100) at 330 nm are presented.

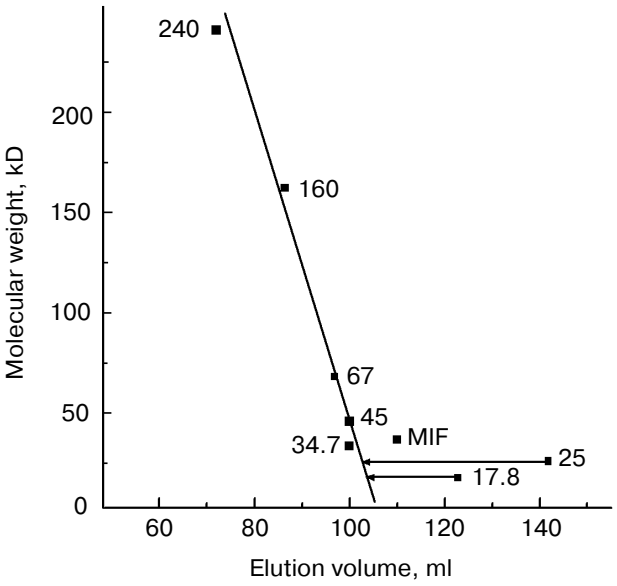

Fig. 2. Calibration curve of molecular weights versus elution volume of the standard molecular weight markers in 20 mM Tris-HCl buffer (pH 7.0) (catalase, 240; aldolase, 160; BSA, 67; ovalbumin, 45; pepsin, 34.7; chymotrypsinogen, 25; myoglobin, 17.8 kD). The peak yield of MIF occurs at 110 ml. The arrows indicate yield volumes of the markers, chymotrypsinogen and myoglobin, using buffer with high ionic strength.

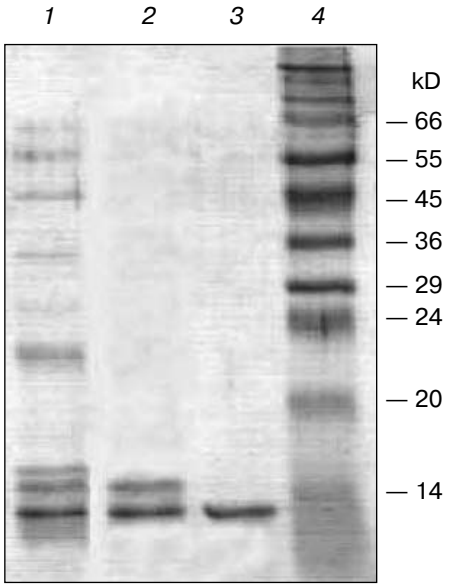

Fig. 3. SDS-PAGE of MIF-containing fractions obtained at different stages of the MIF isolation. Lanes: *1*, *2*) in the eluate from the Toyopearl HW-55 column in 20 mM Tris-HCl buffer (pH 7.0) containing 2 mM β-ME in the presence or absence of 0.2 M KCl, respectively; *3*) the same in 20 mM Tris-HCl buffer (pH 7.0) without KCl and β-ME; *4*) standard molecular weight markers, kD. The bands were developed with silver staining.

increased and was revealed in a significantly greater volume (from 100 to 120 ml) (Fig. 1, curve *2*) when 20 mM Tris-HCl buffer (pH 7.0) containing 2 mM β-ME was used as the eluent.

To determine the mechanism of MIF interaction with the sorbent of the column, in a separate series of experiments eluting buffer with increased ionic strength due to addition of 0.2 M KCl was used. This slightly decreased the sorption ability of the sorbent. Salt is often added into the buffer for gel filtration with different media to decrease protein sorption on a column and increase its yield.

The MIF-containing fractions eluted from the column with Toyopearl HW-55 were subjected to SDS-electrophoresis in polyacrylamide gel, and the MIF was homogenous when the buffer with low ionic strength without β-ME was used. The electrophoregram presented a single band, which corresponded to an apparent molecular weight of 12 kD (Fig. 3, lane *3*).

To prove the identity of the isolated protein as MIF, immunoblotting with a PVDF membrane was performed after electrophoresis (Fig. 4). The immunoreactive band was cut out and microsequenced. The resulting N-terminal sequence (29 steps) was identical to the MIF structure: NH_2-PMFVVNTNVP RASVPDGLLS ELTQQLAQA. According to the Databank of protein structures, this amino acid sequence is identical to the N-terminal structure of the MIF molecule from calf brain consisting of 114 amino acid residues [37, 38].

But the use of the buffer containing 0.2 M KCl and/or 2 mM β-ME resulted in loss of homogeneity (Fig. 3, lanes *1* and *2*). Under these conditions, alkaline proteins of the bovine brain extract and isolated concomitantly with MIF can markedly lose the ability of being adsorbed on the column, as occurs in the case of chymotrypsinogen, a 25-kD marker protein (Fig. 2). The presence of β-ME can lead to the appearance in the MIF-containing fractions of other proteins, which in the absence of β-ME were constituents of higher molecular weight structures and were eluted earlier than MIF (Fig. 3, lane *2*). However, the results of immunoblotting (Fig. 4, lane *1*) suggest that the concomitant fractions contain no MIF-like proteins because they do not react with anti-MIF antibody.

The Japanese firm Toyo Soda describes the sorbent Toyopearl as a hydrophilic vinyl polymer containing numerous hydroxyl groups. Toyopearl is also characterized by the ability of adsorbing aromatic compounds. In the present work these properties were, in particular, manifested by the sorption on the column of an alkaline protein (p*I* 9.5) chymotrypsinogen (a 25-kD molecular weight marker) (Fig. 2). Obviously, at pH 7.0 this protein has a net positive charge, which promotes its interaction with hydroxyl groups of the sorbent.

The delayed elution of MIF because of its interaction with hydroxyl groups of the sorbent is unlikely. By isoelectric focusing under native conditions, we have shown that the isoelectric point of MIF is 7.1. Moreover, increase in the ionic strength of the eluting buffer 20 mM

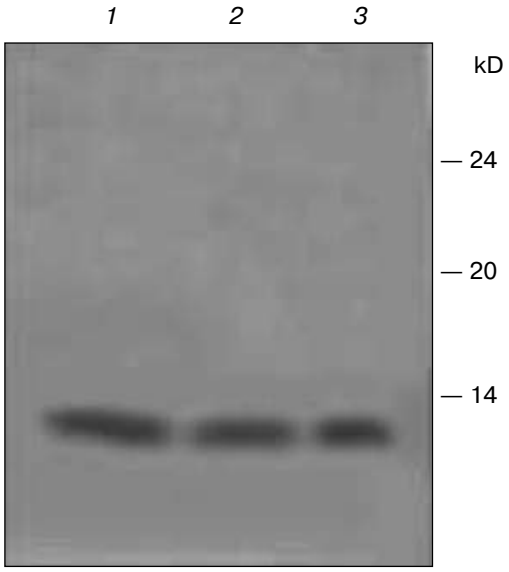

Fig. 4. Immunoblotting of MIF-containing fractions eluted from the Toyopearl-HW-55 column with 20 mM Tris-HCl buffer (pH 7.0) without KCl in the presence (*1*) or absence of 2 mM β-ME in the activity peak (*2*) and in the right shoulder (*3*). To the right standard molecular weight markers are given. Commercial anti-MIF antibody (R&D Systems) was used.

Tris-HCl (pH 7.0) by addition of 0.2 M KCl only slightly displaced to the left the peak of MIF activity.

Most likely, MIF is adsorbed on the column due to the presence in its molecule of aromatic amino acid residues. X-Ray diffraction data [29] show that aromatic amino acids (Tyr37, Phe50, Tyr96, Trp109, and Phe114) form a hydrophobic cluster on the surface of MIF.

Even in the earliest studies on the tautomerase activity of MIF, it was shown to be a thiol-dependent enzyme [34]. Later the primary structure of MIF was found to include three cysteines, two of which (Cys57 and Cys60) form a single intramolecular disulfide bridge [25]. However, experiments with mutant MIF with Cys57 and Cys60 substituted by Ser have shown that these substitutions do not affect the keto–enol tautomerase activity of MIF. But the replacement of the third cysteine (Cys81) by Ser resulted in more than 30% decrease in the enzymatic activity [25] that was caused by conformational changes in the MIF molecule [39]. In our experiments the elution of MIF with the buffer containing 2 mM β-ME nearly threefold increased its tautomerase activity (Fig. 1, curve *2*). A similar result was obtained on addition of β-ME into the MIF preparation isolated in the absence of β-ME.

The proposed easy and rapid method for MIF isolation gives a relatively high yield of the biologically active protein (0.1 mg homogenous MIF per g wet brain tissue, i.e., 40 mg homogenous MIF per liter of the protein suspension in ammonium sulfate). And the protein is purified about 200-fold compared to the supernatant of the bovine brain homogenate (60,000*g*, 1 h).

On the isolation of MIF as described in [40] (the modified scheme of [41]), the bovine brain extract was purified by anion-exchange chromatography on a column with DEAE-Servacel. The MIF-containing fractions were subjected to reversed-phase HPLC, and MIF was detected in two fractions (which were eluted at 57 and 60% acetonitrile) that indicated a high hydrophobicity of MIF. N-Terminal sequences of amino acid residues of proteins in these fractions (43 and 26 steps, respectively) were identical to the above-presented structure of MIF. However, during SDS-PAGE each of the fractions was manifested as two bands with apparent molecular weights of 12 and 29 kD, whereas on HPLC they were eluted from the column as a single peak. Sequencing of these proteins revealed the MIF-like primary structure [40]. This scheme of isolation was advantageous because it allowed us to prepare MIF isoforms.

Isoforms of MIF could not be revealed by the method described in this paper. We suggest that on exclusion chromatography one of the isoforms can occur in heterooligomers eluted in the range of high-molecular-weight fractions, because we have earlier shown their immune reactivity to anti-MIF antibody [40].

Although MIF was first detected about 40 years ago, homogenous MIF was isolated from mammalian tissue only in 1993, when the primary structure of MIF from the calf brain was reported [37, 38]. The human recombinant MIF (MIFhr) prepared in 1989 [3] is now the most popular object for investigations. The purification of MIFhr expressed in *Escherichia coli* by two-step ion-exchange chromatography and subsequent gel filtration yielded ~40 mg homogenous MIF per liter of the cell suspension [29], which correlates with our results. Later MIFhr was reported to be isolated from *E. coli* cells by gel chromatography on Sephadex G-50 and subsequent cation-exchange chromatography on carboxymethyl cellulose, which improved the yield of the pure protein [42]. But this method is more tedious than the approach proposed above.

The MIF content and distribution in tissues, biological fluids, and cells of mammals in norm and disease are mainly studied by immunocytochemical methods that cannot identify structural isoforms and oligomeric states of MIF, which is necessary for understanding mechanisms of its action. Results of the present work can be used for isolation and identification of MIF also from other sources, and this will extend our knowledge about this unique polyfunctional protein.

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